

# Separation of *Mycobacterium lepraemurium* from the Subcutaneous Tissues of the Rat<sup>1</sup>

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In order to achieve certain objectives in biochemical and metabolic studies, it is essential to collect *M. leprae* or *M. lepraemurium* which are free from tissue components and blood cells. It is also important not to subject the tissues to such chemicals or reagents which might have deleterious effects on the bacilli. Several methods have been described for the isolation of *M. leprae* and *M. lepraemurium* from host tissues by a number of investigators. Dharmendra (<sup>1</sup>) and Khanolkar and Rajalakshmi (<sup>3</sup>) reported that the bacilli can be isolated from host tissues by chloroform or ether extraction. In these methods the bacteria are subjected to reagents which may change not only their viability but also their physical and chemical characteristics as well. Moreover, during these procedures, large numbers of the bacilli are lost in the discarded tissues. Lew and Carpenter (<sup>4</sup>) prepared a suspension of tissue-free *M. lepraemurium* by treating lepromatous tissues with trypsin for two hours at 37°C. Nakamura (<sup>6</sup>) also collected intact bacilli by treating the tissues with trypsin. Hanks' method (<sup>2</sup>) appears to be adequate except that disintegration of the infected organs in a Waring blender for two minutes and further homogenization for five minutes may cause some damage to the bacilli. Likewise, the method of Mori *et al* (<sup>5</sup>), which involves several centrifugation steps, also seems to be promising for collecting *M. lepraemurium*, except that treatment with N/8 NaOH may have some undesirable effects upon the bacilli.

Our recent spectral studies indicated that the presence of only traces of blood cells in the bacillary suspensions may give erroneous results. In the present report, a method for collecting relatively large quantities of intact *M. lepraemurium* cells is described

and spectrophotometric evidence is provided for the purity of bacillary suspensions.

## MATERIALS AND METHODS

**Preparation of purified cell suspensions.** The Hawaiian strain of rat leprosy was maintained in Sprague-Dawley rats by serial transmission at three to four month intervals. Four to six large lepromata were removed aseptically and cut into small pieces with a scissors and rinsed thoroughly with sufficient distilled water to remove loose tissues and excess blood, if any. They were homogenized in cold 0.1 M potassium phosphate buffer, pH 7.4, in a Servall Omnimixer five times for three seconds at 12,000 rpm. The homogenized material was filtered through a sterile nylon filter and the host tissues retained on the filter were washed three to four times with small amounts of phosphate buffer. The filtrate, containing mainly bacilli, few connective tissue fibers, inert fragments and small amounts of blood cells, was transferred into transparent polycarbonate round bottom bottles and centrifuged at 500 × g for eight minutes in a JS-7.5 swinging bucket rotor. The supernatant fluid was decanted and the sediment containing blood cells and connective tissue fibers was washed twice with phosphate buffer, pH 7.4, at low speed (500 × g) centrifugation. The pooled supernatant was centrifuged in round bottom translucent polycarbonate tubes at 12,000 × g for eight minutes. The debris contained a very small amount of reddish pellet underneath the sedimented bacilli. The bacillary sediment was very carefully washed off by means of a sterile hypodermic syringe, leaving behind the reddish pellet which consisted mainly of blood cells. The blood cells were washed twice and then resuspended in buffer for spectral analysis. This suspension will subsequently be referred to as the blood cell suspension. As will be shown later, it was absolutely necessary to eliminate this small amount of blood cells. The pooled supernatant, containing bacilli, was centrifuged at 12,000 × g for ten minutes and the

<sup>1</sup> Received for publication 9 October 1973.

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resulting pellet was suspended in a small volume of buffer. All washings were carried out at 4°C in a Beckman Model J-21B refrigerated centrifuge. Sterile equipment and 0.1 M potassium phosphate buffer, pH 7.4, were used under aseptic conditions throughout this study.

**Spectrophotometric determination.** The difference spectra<sup>3</sup> of suspensions were obtained in a Unicam SP ultraviolet dual beam recording spectrophotometer equipped with an expanded scale of 0-0.2 OD. All spectra were taken at room temperature and pyrex glass cuvettes 4 mm wide with a light path of 10 mm were used. The anaerobically-re-

duced minus O<sub>2</sub>-oxidized difference spectra of whole cell suspensions of *M. lepraemurium* were recorded immediately after evacuating the treatment cuvette (Thunberg type) without added substrate and bubbling oxygen in the reference cuvette for three to five minutes. The dithionite-reduced minus O<sub>2</sub>-oxidized difference absorption spectra of bacillary suspensions was obtained after the cell suspensions in the treatment cuvette were treated with dithionite and the reference cuvette was aerated with oxygen. Difference spectra of blood cell suspensions were obtained by the addition of dithionite in the treatment cuvette.

## RESULTS

It was frequently observed that different lots of cell suspensions of *M. lepraemurium*

<sup>3</sup>A difference spectrum represents the differences of optical densities of the reduced and oxidized forms of the pigments.

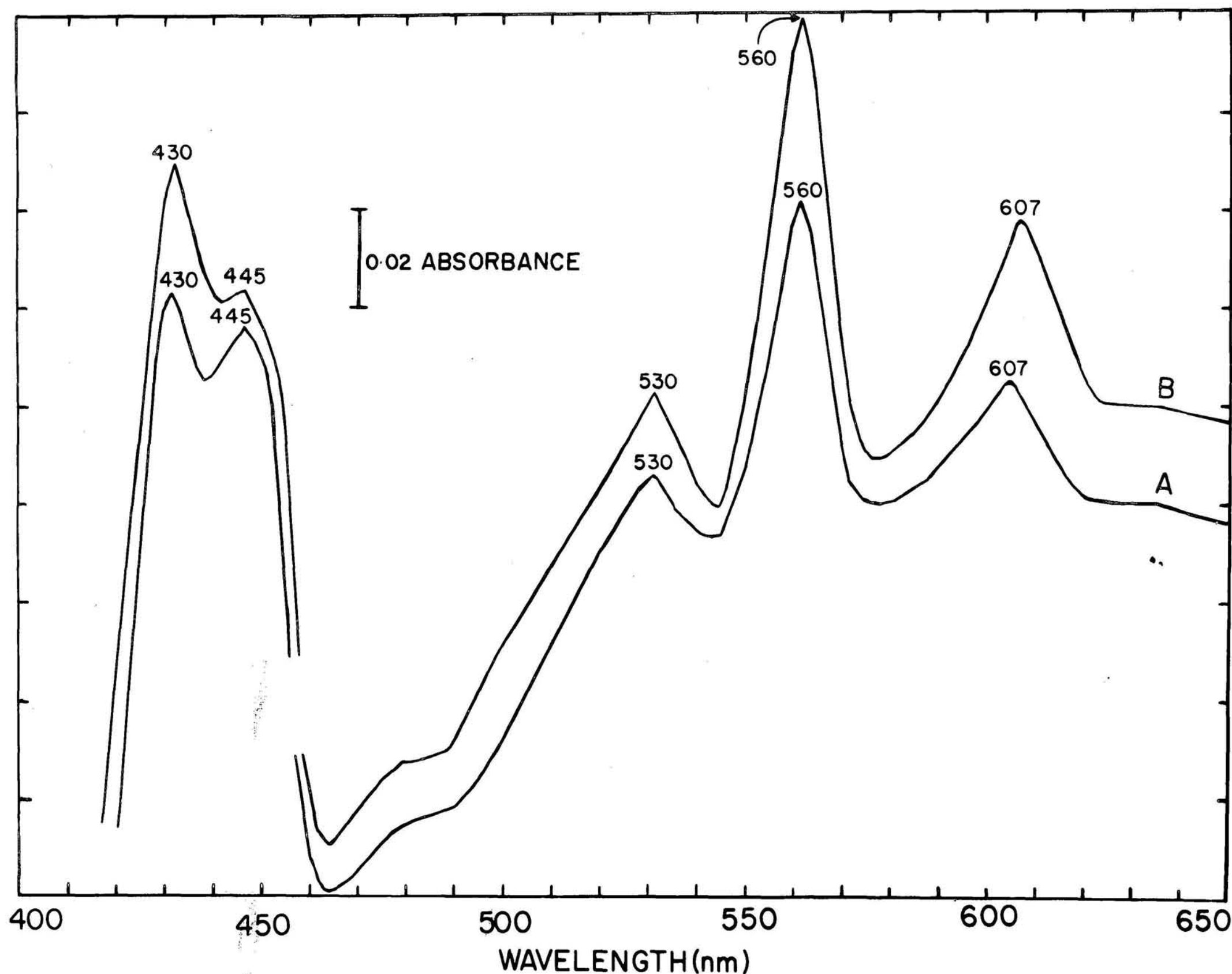


FIG. 1. Difference absorption spectra of whole cells of *M. lepraemurium*. Reaction mixture in a total volume of 2 ml contained 20 mg (dry weight) of whole cells in 0.1 M potassium phosphate buffer, pH 7.4. Trace A represents absorption spectrum taken immediately after oxygen was passed for five minutes through the reference cuvette and the sample cuvette was made anaerobic without added substrate. Trace B shows dithionite-reduced minus O<sub>2</sub>-oxidized difference spectrum. Cell suspension was diluted twofold for measurements in the Soret region (400-450 nm) of the spectrum.

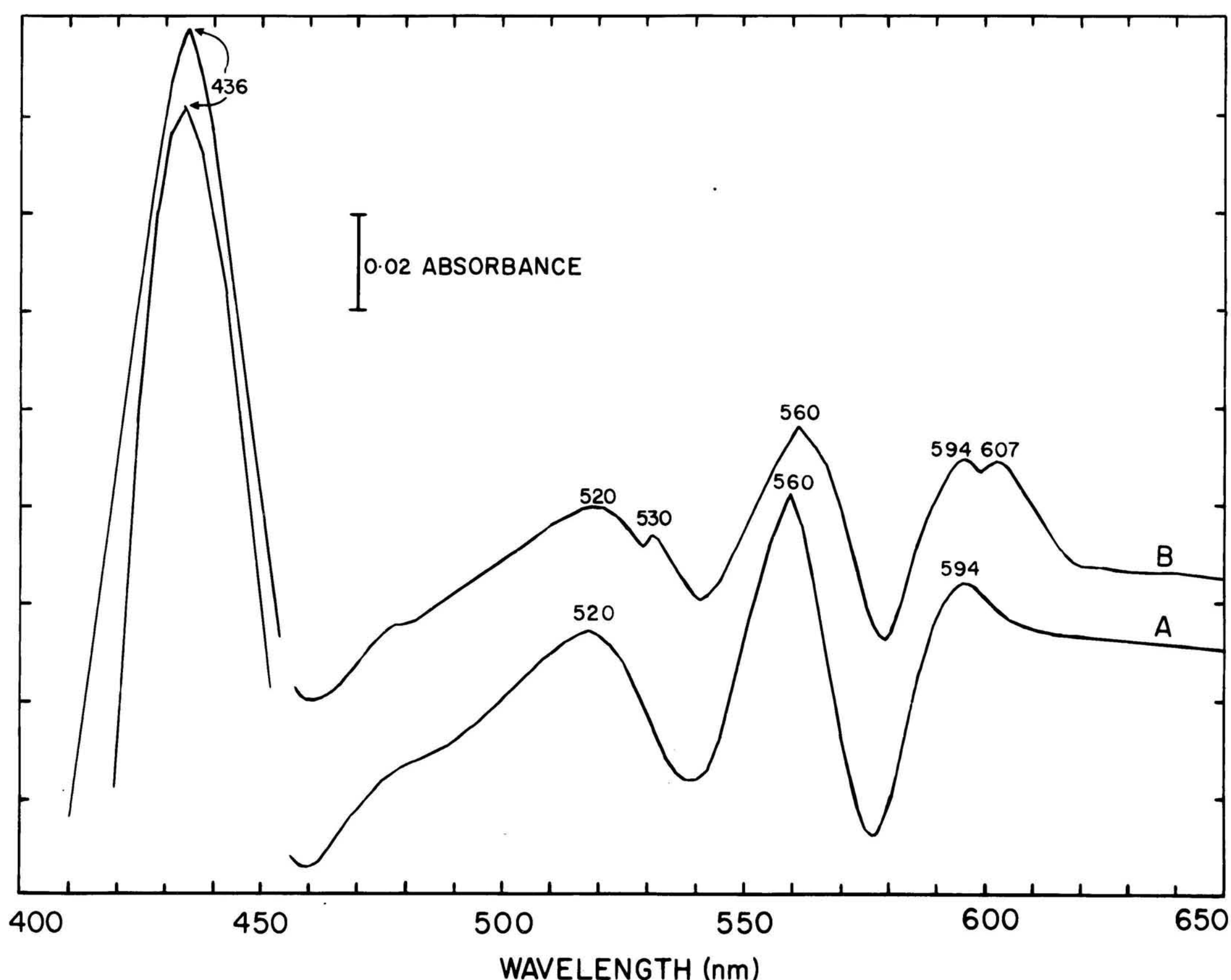


FIG. 2. Difference spectra of blood cell suspensions and *M. lepraemurium* cell suspensions contaminated with blood cells. Trace A indicates dithionite-reduced minus oxidized difference spectrum of blood cells (4 mg dry weight) in 2 ml of 0.1 M phosphate buffer (pH 7.4). Trace B represents dithionite-reduced minus oxidized absorption spectrum of mycobacterial cell suspensions (18 mg dry weight) contaminated with blood cells (2.5 mg dry weight) in 2 ml of 0.1 M phosphate buffer, pH 7.4. Cell suspension was diluted twofold for measurements in the Soret region (400-450 nm) of the spectrum.

gave different reduced minus oxidized absorption spectra. It became evident that this difference in spectra was caused by small amounts of blood cells present in some preparations. We have observed that whole cell suspensions of *M. lepraemurium* contained cytochrome systems which were all in the reduced form. The anaerobically-reduced (in the absence of added substrate) minus  $O_2$ -oxidized difference spectra of purified, intact mycobacterial cell suspensions is shown in Figure 1. This spectrum indicates the presence of *b*-type cytochrome with maxima at 430, 530 and 560 nm (nanometer), as well as of *a* + *a*<sub>3</sub> type cytochromes with peaks at 445 and 607 nm (Trace A). The peaks in the dithionite-reduced minus  $O_2$ -oxidized difference spectrum of purified cell suspensions of *M. lepraemurium* (Trace B) were somewhat

larger but they also suggested the presence of the same type of cytochromes. Anaerobically or dithionite-reduced minus  $O_2$ -oxidized difference spectra gave no evidence for the presence of cytochrome *c* in intact cells of *M. lepraemurium*.

Unlike the whole cell suspensions of *M. lepraemurium*, the cytochrome systems of the blood cell suspensions were mainly in the oxidized form and could not be reduced by the endogenous substrate. Moreover, the difference spectra of blood cell suspensions was quite different from that of purified whole cell suspensions of *M. lepraemurium*. The addition of dithionite in the blood cell suspensions caused the appearance of small absorption bands at 594 and 520 nm and distinct peaks at 560 and 436 nm (Fig. 2, Trace A). The dithionite-reduced minus oxidized



difference spectrum of whole cell suspensions contaminated with blood cells (Fig. 2, Trace B) revealed peaks at 607, 594, 560, 520 and 436 nm. The peaks in the alpha and beta region were those exhibited by both the purified cell suspensions (Fig. 1) as well as by the blood cell suspensions (Fig. 2, Trace A). However, the peaks in the Soret region at 430 and 445 nm of purified cell suspensions were masked by a distinct peak of blood cell suspensions at 436 nm.

### DISCUSSION

The criteria used to determine the purity of the isolated bacillary suspensions include Ziehl-Neelsen staining, viability, infectiousness and electron microscopic examination. As the standard method for the collection of *M. leprae* and *M. lepraemurium* is still undecided, each investigator has his own method of isolating leprosy bacilli without tissue components for experimental purposes. It is less important that the isolated cell suspensions are 100% pure but the main object of the method must be to eliminate the contaminants which interfere in specific studies. For example, our spectrophotometric results show that the presence of a small amount of blood cells in the cell suspensions may give misleading results. As shown in Figure 1, the purified whole cell suspensions always resulted in difference spectra which showed peaks of cytochrome  $a + a_3$  (607 and 445 nm) and cytochrome  $b$  (560, 530 and 430 nm). However, the difference spectra of the preparations contaminated with small amounts of blood cells revealed peaks of both the purified cell suspensions as well as of blood cell suspensions (Fig. 2, Trace B). Such suspensions were considered unsuitable for spectrophotometric studies and were further purified or discarded. Electron microscopic examination revealed that insufficiently purified cell suspensions always contained traces of blood cells and the preparations of more than 95% purity were free from blood cells.

### SUMMARY

In an effort to obtain cell suspensions free from blood cells which interfere in spectrophotometric studies, a method was devised by which relatively large quantities of whole cell suspensions of *M. lepraemurium* are obtained for experimental purposes. We have

routinely employed this procedure which is quite reliable, technically simple, moderate in equipment requirements, and in a matter of five minutes it can be ascertained whether or not the preparations are free from contaminants which render the purified bacillary suspensions unsuitable for studies involving spectrophotometric technics. However, in this procedure, certain points are essential in order to obtain large quantities of purified bacillary suspensions. It is important to homogenize the lepromata for only a few seconds at brief intervals. Excessive homogenization may cause considerable damage to the bacilli and it may be difficult to separate the bacilli from the host tissues. It is also important to use transparent bottles and tubes during centrifugation as the material can be seen and thus each fraction can be separated easily. Very few bacilli are lost in the discarded material and we have repeatedly obtained 2.5 to 3 gm wet weight of *M. lepraemurium* from one leproma weighing 25 gm to 30 gm.

### RESUMEN

En un intento para obtener suspensiones de células libres de células sanguíneas, que interfieren con los estudios espectrofotométricos, se desarrolló un método por medio del cual se obtienen relativamente grandes cantidades de suspensiones de células intactas de *M. lepraemurium* para trabajos experimentales. Hemos empleado este procedimiento en forma rutinaria, ya que es bastante seguro, técnicamente simple, moderado en lo que respecta a necesidades de equipo, y en escasos cinco minutos se puede determinar si las preparaciones están libres de los contaminantes que hacen que las suspensiones bacilares purificadas no sean adecuadas para estudios que incluyen la utilización de técnicas espectrofotométricas. Sin embargo, en este procedimiento hay ciertos puntos que son esenciales para poder obtener grandes cantidades de suspensiones bacilares purificadas. Es importante homogeneizar los lepromas durante sólo algunos segundos, a cortos intervalos. La excesiva homogeneización puede producir considerable daño a los bacilos y puede dificultar la separación de los bacilos del tejido del huésped. También es importante usar botellas y tubos transparentes durante la centrifugación del material, para que este se pueda ver y cada fracción pueda separarse fácilmente. Se pierden muy pocos bacilos en el material que se descarta y repetidamente hemos obtenido 2,5 a 3 gm de peso húmedo de *M. lepraemurium* de una leproma que pesaba entre 25 y 30 gm.



## RÉSUMÉ

En vue d'obtenir des suspensions cellulaires libres de cellules du sang, susceptibles d'interférer dans les études spectrophotométriques, on a mis au point une méthode grâce à laquelle on peut obtenir, à des fins expérimentales, des suspensions de cellules entières de *M. lepraemurium*. Ce procédé a été employé de manière routinière. Il s'est révélé tout à fait fiable, simple sur le plan technique, et par contre n'a requis qu'un équipement relativement réduit. Il suffit de cinq minutes pour se rendre compte si une préparation est indemne ou non des contaminants qui peuvent rendre des suspensions bacillaires purifiées impropres aux études qui exigent des techniques spectrophotométriques. Il est cependant essentiel, dans cette technique, de respecter certains points, afin d'obtenir de grandes quantités de suspensions bacillaires purifiées. Il est important d'homogénéiser les léprômes pour quelques secondes, et pas plus, à de brefs intervalles. Une homogénéisation excessive peut entraîner une détérioration considérable des bacilles, telle qu'il devient difficile de les séparer des tissus hôtes. Il est également important d'utiliser des flacons et des tubes transparents pour la centrifugation, afin que le matériel puisse être observé, ce qui facilite la séparation de chaque fraction. Très peu de bacilles sont perdus dans le matériel rejeté. De façons répétées, on a pu obtenir 2,5 à 3 gm de poids humide de *M. lepraemurium*, à partir d'un léprôme pesant 25 à 30 grammes.

**Acknowledgments.** This research was supported by National Health Grant Nos. 604-7-818 and 604-7-821 from the Department of Health and Welfare, Canada, and a generous donation from the Institute Fame Pereo.

## REFERENCES

1. DHARMENDRA. Studies on the lepromin test. 3. Preparation and standardization of lepromin. *Lepr. India* 13 (1941) 77-80.
2. HANKS, J. H. Measurement of the hydrogen transfer capacity of mycobacteria. *J. Bacteriol.* 62 (1951) 521-528.
3. KHANOLKAR, V. R. and RAJALAKSHMI. A method for concentration of acid-fast bacilli in skin biopsies from leprosy patients. *Lepr. India* 24 (1952) 47-61.
4. LEW, J. and CARPENTER, C. M. The separation of *M. leprae* from tissues by enzyme digestion. *Am. Rev. Tuberculosis* 74 (1956) 156.
5. MORI, T., KOHSAKA, K. and TANAKA, Y. Tricarboxylic acid cycle in *M. lepraemurium*. *Int. J. Lepr.* 39 (1971) 796-812.
6. NAKAMURA, M. Attempts to approach the clarification of chemical composition of *M. lepraemurium*. 1. Separation of the bacilli from the subcutaneous tissue of rat inoculated with *M. lepraemurium* and some properties of the bacilli. *Kurume Med. J.* 2 (1955) 110-116.